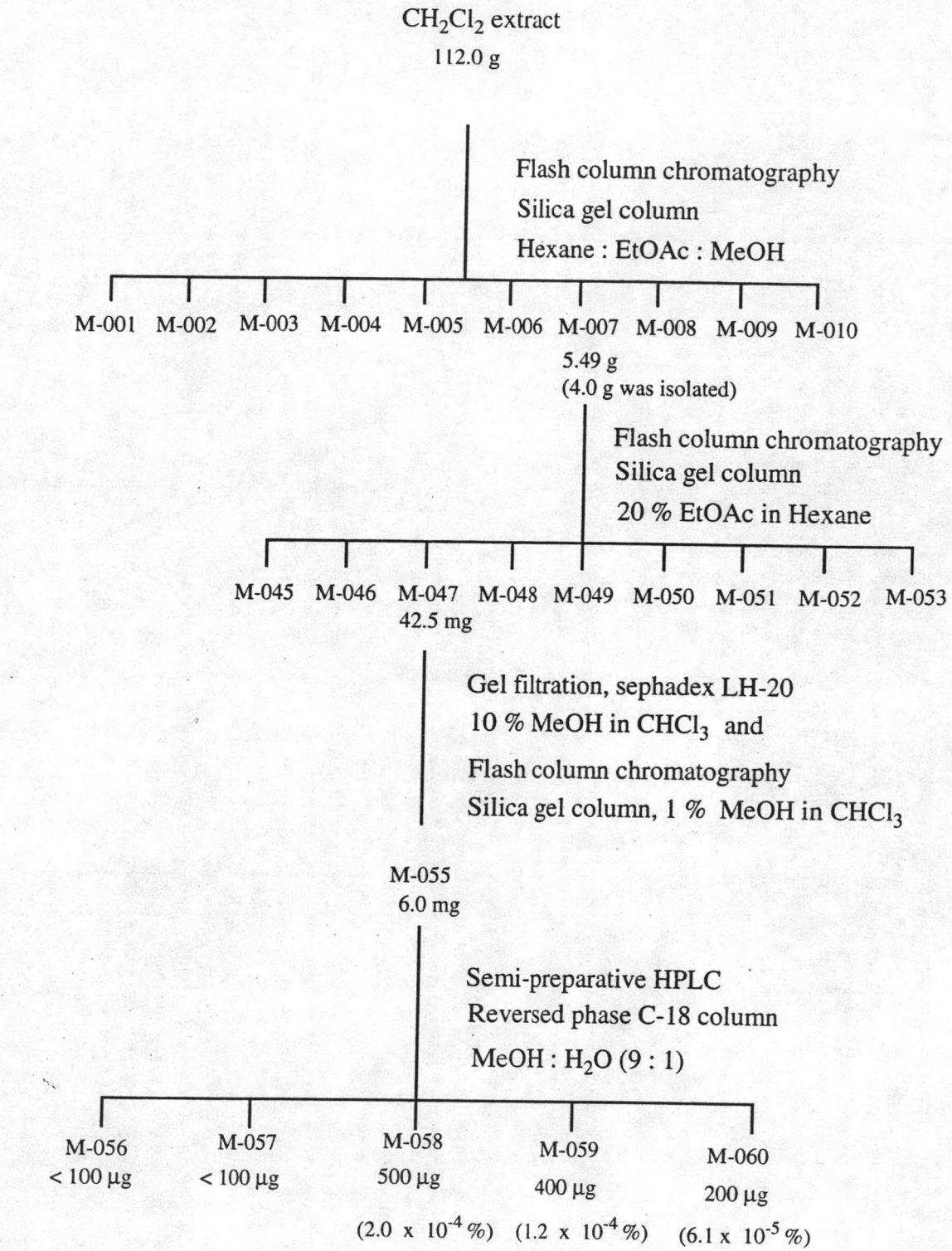
CHAPTER IV

RESULTS AND DISCUSSION

Isolation of Chemical Constituents

1. Isolation of chemical constituents from the dichloromethane extract.

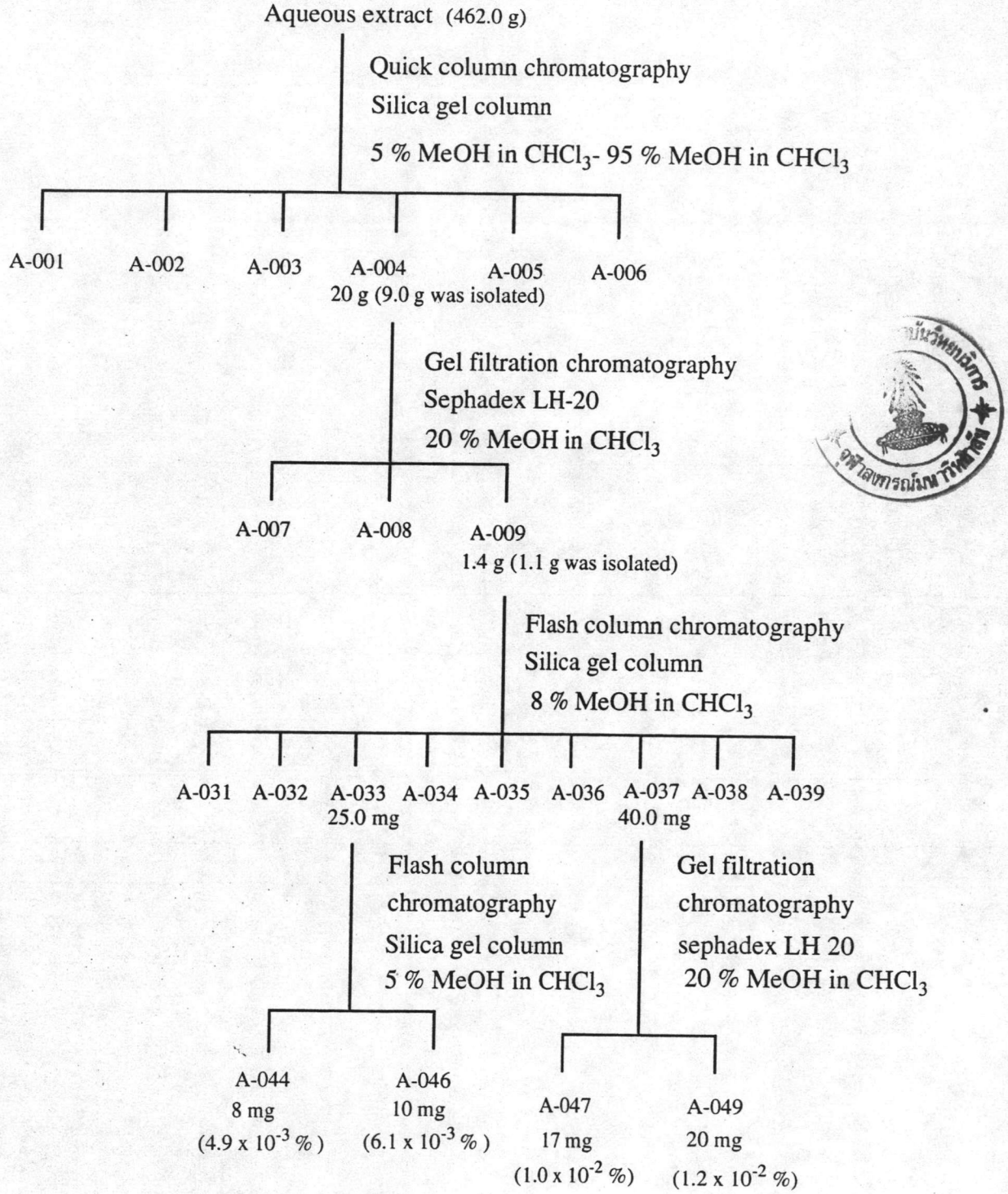
The dichloromethane extract from *Mycale* sp. showed cytotoxic activity against murine leukemia cell (P-388), human lung carcinoma (A-549), human colon carcinoma (HT-29) at IC_{50} = 0.26-0.6 μg/ml. The crude dichloromethane extract (112.0 g) was fractionated by flash column chromatographic technique to yield ten fractions (M-001-M-010). The major components of fractions M-004-M-006 were known sesterterpene cyclic-peroxides, mycaperoxides A and B (Tanaka *et al.*, 1993). Mycaperoxides A and B produced brown color with anisaldehyde sulphuric acid reagent but did not show quenching spot under uv light at λ 254 nm. The other fractions M-001-M-003 and M-007-M-010 were sent to test cytotoxic activity. M-007 (5.49 g) was the most active fractions IC_{50} = 0.2 μg/ml (P-388, A-549, and HT-29 cell lines). TLC chromatogram of M-007 (Figure 9) showed three quenching spots under uv light at λ 254 nm. One quenching spot (R_f = 0.61) produced brown color with anisaldehyde sulphuric acid reagent. TLC chromatogram of M-007 (Figure 10) also showed other brown spot after spraying with anisaldehyde sulphuric acid (R_f = 0.32, non quenching). The latter spot (R_f = 0.32) was mycaperoxides. M-007 was fractionated by flash column chromatographic technique to obtain fractions M-045-M-053. Fraction M-047 (42.5 mg) contained quenching spot which produced brown color with anisaldehyde sulphuric acid reagent. Fraction M-047 was further fractionated by gel filtration technique and flash column chromatographic technique to yield fraction M-055 (6 mg). Solution of M-055 in methanol was injected to semi-preparative hplc C-18 column to yield compounds M-056 (< 100 μg), M-057 (< 100 μg), M-058 (500 μg), M-059 (400 μg) and M-060 (200 μg). HPLC chromatogram is summarized in Figure 11. The isolation diagram is shown in Scheme 1.



Scheme 1. Isolation diagram of M-059 and M-060

2. Isolation of chemical constituents from the aqueous extract.

The aqueous extract (462.0 g) from *Mycale* sp.was fractionated by quick column chromatographic technique to yield A-001-A-006. A-004 (9.0 g) was fractionated by gel filtration technique to yield A-007-A-009. A-009 (1.38 g) was further fractionated by flash column chromatographic technique to yield A-031-A-039. A-033 (25.0 mg) showed two quenching spots under uv light at λ 254 nm. These two spots did not produced blue color with anisaldehyde sulphuric acid. Fraction A-033 was isolated by the flash column chromatographic technique to yield A-044 (Rf = 0.46, 8.0 mg) and A-046 (Rf = 0.34, 10.0 mg). A-037 (40.0 mg) showed two quenching spots under uv light at λ 254 nm. These two spots were produced blue color with anisaldehyde sulphuric acid. A-037 was further isolated by gel filtration technique to yield A-047 (Rf = 0.24, 17.0 mg) and A-049 (Rf = 0.16, 20.0 mg). TLC chromatogram of A-033 and A-037 is shown in Figure 12. The isolation diagram is shown in Scheme 2.



Scheme 2. Isolation diagram of A-044, A-046, A-047, and A-049

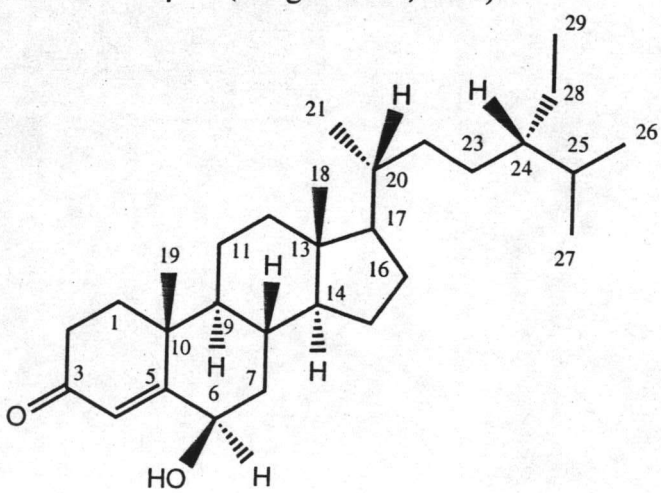
The Stucture Elucidation of The Isolated Compounds.

1. Structure elucidation of the isolated steroids

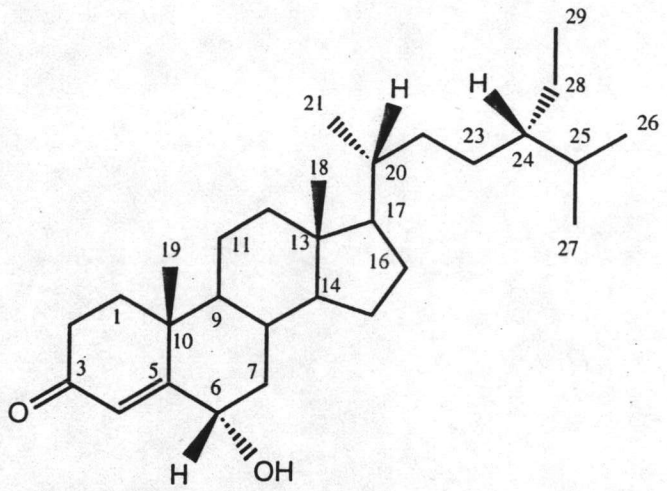
1.1 Compound M-060

Compound M-060, a white compound was obtained from fraction M-055 by semipreparative C-18 hplc column using 10 % water in methanol as a mobile phase. This compound shows uv absorption (Figure 13) in chloroform at λ_{max} 243 nm (ε_{max} = 16,653) which is characteristic of α, β unsaturated ketone. The ir spectrum of M-060 (Figure 14) shows absorption at 3,431 cm^{-1} (OH stretching), 2,918 cm^{-1} (C-H stretching), 1671 cm^{-1} (unsaturated carbonyl stretching), and 1,645 cm^{-1} (C=C stretching). The electron impact mass spectrum (Figure 39) shows the molecular ion peak at m/z = 428 (10.63 %) and establishes the tentative molecular formula of $C_{29}H_{48}O_2$.

M-060 can be assigned as a new steroidal ketone, (24 *S*)-24-ethylcholest-4-en-3-one-6β-ol or poriferast-4-en-3-one-6β-ol, [108] by analysis of its ^1H and ^{13}C nmr spectral data and also comparing with procesterol or [(24*S*)-24-ethylcholest-4-en-3-one-6α-ol], [104] (Khan and Malik 1989), (24 *R*)-24-ethylcholest-5-en-3β-ol, and (24 *S*)-24-ethylcholest-5-en-3β-ol (Wright *et al.*., 1978).



[108]



[104]

The ^{1}H nmr spectrum of M-060 (Figures 15, 16, 17, and 18) shows six methyl groups at δ 1.38 ppm (s), δ 0.93 ppm (d, J = 6.6 Hz), δ 0.86 ppm (t, J = 7.4 Hz), δ 0.83 ppm (d, J = 6.8 Hz), δ 0.81 ppm (d, J = 7.0 Hz), and δ 0.74 ppm (s) which is the characteristic of 19-CH_3, 21-CH_3, 29-CH_3, 27-CH_3, 26-CH_3, and 18-CH_3, of the C-29 steroid, respectively. In addition, the spectrum shows an olefinic proton signal at δ 5.82 (s) and a methine proton connected to the oxygenated carbon at δ 4.34 (br.t, J = 2.9 Hz).

The ^{13}C nmr spectrum of M-060 (Figures 19, 20) shows 29 carbons. The carbon types can be classified by Distortionless Enhancement by Polarization Transfer (DEPT) experiment, the DEPT 90 spectrum (Figure 21) shows signals of methine carbon while DEPT 135 spectrum (Figure 22) shows positive signals of methine and methyl carbons and negative signals of methylene carbons. From this experiment 29 carbons of M-060 can be assigned as 4 quaternary carbons at δ 200.44 ppm, δ 168.49 ppm, δ 42.54 ppm, and δ 38.02 ppm, 9 methine carbons at δ 126.36 ppm, δ 73.21 ppm, δ 56.08 ppm, δ 55.92 ppm, δ 53.66 ppm, δ 46.10 ppm, δ 36.26 ppm, δ 29.76 ppm, δ 29.00 ppm, 10 methylene carbons at δ 39.63 ppm, δ 38.54 ppm, δ 37.13 ppm, δ 34.28 ppm, δ 33.91 ppm, δ 28.18 ppm, δ 26.44 ppm, δ 24.18 ppm, δ 23.06 ppm, δ 21.01 ppm, and 6 methyl carbons at δ 19.61 ppm, δ 19.53 ppm, δ 19.00 ppm δ 18.80 ppm, δ 12.32 ppm, and δ 12.03 ppm The signals at 200.44, 168.49 and 126.36 ppm are the carbons of an enone moiety, supported by the unsaturated carbonyl stretching in the ir spectrum.

The structure of M-060 is completely determined by several technique in two dimensional nmr experiments, including High Sensitive-Quantum Coherence (HSQC),

Heteronuclear Multiple Bond Coherence (HMBC) and Phase-Sensitive Double Quantum Frequency Correlated Spectroscopy (PDQF-COSY).

The HSQC spectrum (Figures 23, 24, 25) shows correlation between the directly coupled ^{1}H and ^{13}C nuclei. According to the HSQC experiment of compound M-060, all protons and protonated carbons of M-060 can be assigned. The directly coupled ^{1}H and ^{13}C are summarized in Table 7.

Table 7. The carbon-proton correlations of M-060 observed in the HSQC spectrum

Carbons	δ (ppm)	Correlation with protons at δ (ppm)
C-1	37.13	2.04, 1.71
C-2	34.28	2.52, 2.38
C-4	126.36	5.82
C-6	73.21	4.34
C-7	38.54	2.00, 1.23
C-8	29.76	1.95
C-9	53.66	0.91
C-11	21.01	1.49-1.54
C-12	39.63	2.05, 1.17
C-14	55.92	1.01
C-15	24.18	1.62, 1.15
C-16	28.18	1.86, 1.31
C-17	56.08	1.13
C-18	12.03	0.74
C-19	19.53	1.38
C-20	36.26	1.38
C-21	18.80	0.93
C-22	33.91	1.36, 0.98
C-23	26.44	1.32, 1.04
C-24	46.10	0.94
C-25	29.00	1.68
C-26	19.00	0.82
C-27	19.61	0.83
C-28	23.06	1.33, 1.14
C-29	12.32	0.86

The PDQF spectrum of compound M-060 (Figures 26, 27, 28) shows the correlations of directly coupled protons, therefore the connectivity of the coupled protons can be obtained. PDQF experiment is used to suppress interrupt singlet peaks by using a double quantum filter. The direct ^{1}H-^{1}H coupling observed from the correlations in the PDQF spectrum of compound M-060 are summarized in Table 8 and Figure 5.

The correlations between protons at δ 2.04 ppm and 1.71 ppm (Ha-1 and Hb-1), protons at δ 2.52 ppm and 2.38 ppm (Ha-2 and Hb-2), protons at δ 2.00 ppm and 1.23 ppm (Ha-7 and Hb-7), protons at δ 2.05 ppm and 1.17 ppm (Ha-12 and Hb-12), protons at δ 1.62 ppm and 1.15 ppm (Ha-15 and Hb-15), protons at δ 1.86 ppm and 1.31 ppm (Ha-16 and Hb-16), and protons at δ 1.32 ppm and 1.04 ppm (Ha-23 and Hb-23) confirm the geminal coupling of the methylene protons (2 bond) and support HSQC experiment of methylene groups. Geminal coupling of Ha-11 and Hb-11 at the similar δ 1.49-1.54 ppm, Ha-22 (1.36ppm) and Hb-22 (0.98 ppm), and Ha-28 (1.33 ppm) and Hb-28 (1.14 ppm) can not be observed. The vicinal coupling of protons in four rings and side chain.are observed as following:

Ring A, proton at δ 2.52 ppm (Ha-2) shows correlation to protons at δ 2.04 ppm (Ha-1) and 1.71 ppm (Hb-1). Proton at δ 2.38 ppm (Hb-2) also shows correlation to Ha-1 and Hb-1.

Ring B, proton at δ 4.34 (H-6) shows correlation to protons at δ 2.00 ppm (Ha-7) and 1.23 ppm (Hb-7). Proton at δ 1.95 ppm (H-8) shows correlation to protons at δ 0.91 (H-9) and 1.01 (H-14, ring C). Proton at δ 0.91 (H-9) shows correlation to protons at δ 1.49-1.54 ppm (2H-11, ring C).

Ring C, protons at δ 1.49-1.54 ppm (2H-11) show correlation to proton at δ 1.17 ppm (Hb-12). Proton at δ 1.01 ppm (H-14) shows correlaion to proton at δ 1.62 ppm (Ha-15, ring D).

Ring D, proton at δ 1.62 ppm (Ha-15) show correlation to protons at δ 1.86 ppm (Ha-16), 1.31 ppm (Hb-16) and.1.01 ppm (H-14) Proton at δ 1.15 ppm (Hb-15) shows correlation to Ha-16 at δ 1.86 ppm.

Side chain, proton at δ 1.68 ppm (H-25) shows correlation to protons at δ 0.81 ppm (3H-26), and 0.83 ppm (3H-27).

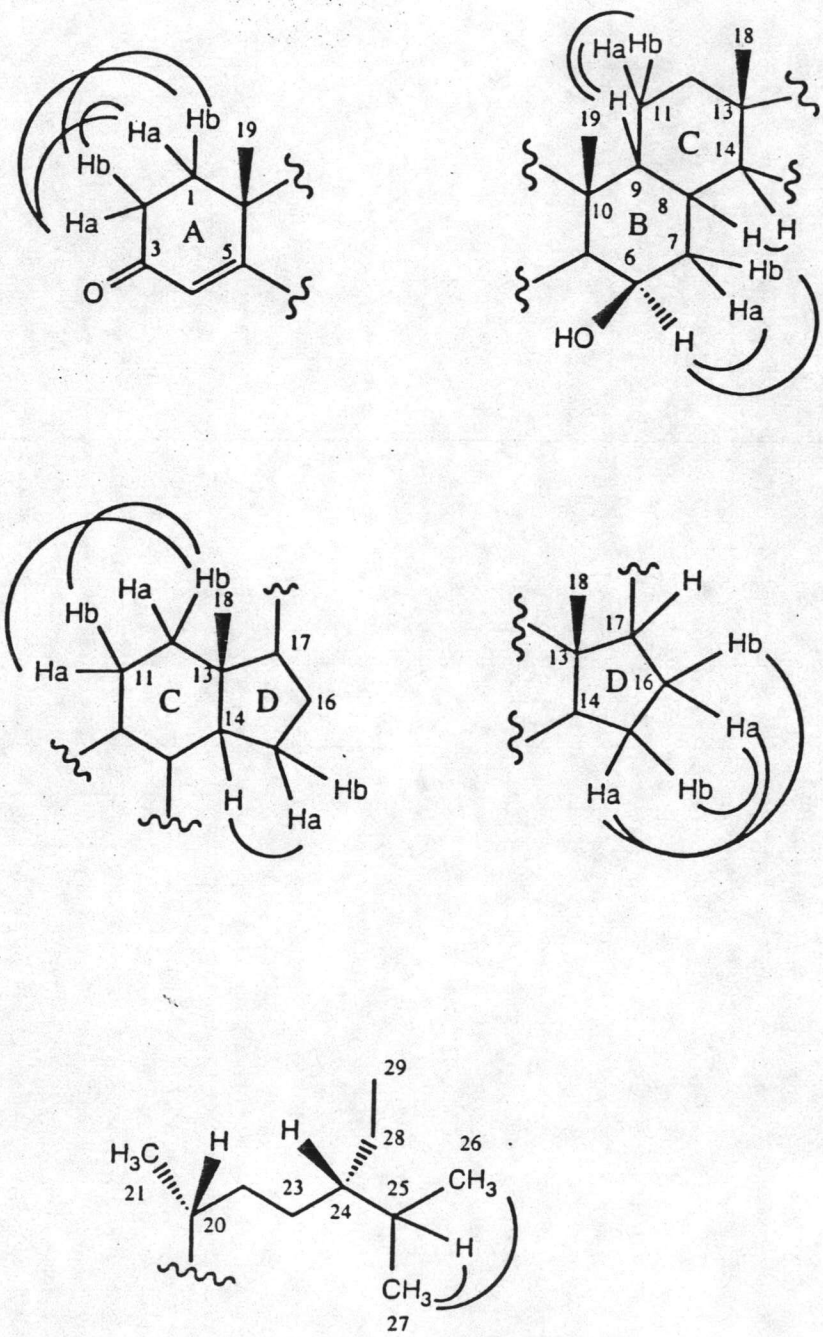


Figure 5. ^{1}H-^{1}H correlation of M-060 observed in PDQF 2-D nmr spectrum

The HMBC spectrum (Figures 29-38) of compound M-060 shows correlations of the long-range coupled ^{1}H and ^{13}C nuclei. The long-range H-C couplings are useful for connecting each molecular fragments together and confirming the placements of the quaternary carbons. The long-range ^{1}H and ^{13}C couplings are shown in Figure 6 and Table 8.

The presence of a 4-en-3-one -6-ol moiety in this compound is proved by the correlations of the olefinic proton, H-4 (δ 5.82 ppm) and C-2 (δ 34.28 ppm), C-6 (δ 73.21 ppm), C-10 (δ 38.02 ppm) ; of Hb-2 (δ 2.38 ppm) and the carbonyl carbon (δ 200.44 ppm) ; of the oxygenated methine proton, H-6 and C-7 (δ 38.54 ppm), C-10 (δ 38.02 ppm). The proton assignment of 19-CH_3 at δ 1.38 ppm is confirmed by its connection to quaternary olefinic carbon C-5 (δ 168.49 ppm).

The connectivity of the side chain at C-17 is supported by the following correlations : 21 CH_3 (δ 0.93 ppm) and C-17 (δ 56.06 ppm), C-20 (δ 36.26 ppm), C-22 (δ 33.91 ppm) ; 26 CH_3 (δ 0.81 ppm) and C-24 (δ 46.10 ppm), C-25 (δ 29.00 ppm), C-27 (δ 19.00 ppm) ; 27-CH_3 (δ 0.83 ppm) and C-24 (δ 46.10 ppm), C-25 (δ 29.00 ppm, C-26 (δ 19.00 ppm) ; 29-CH_3 (δ 0.86 ppm) and C-24 (δ 46.10 ppm), C-28 (δ 23.06 ppm) ; and 28-methylene proton (δ 1.14 ppm, Hb-28) and C-29 (δ 12.32 ppm).

From the above discussion, the structure of compound M-060 is proposed as 24-ethylcholest-4-en-3-one-6-ol steroid. The relative stereochemistry of compound M-060 is determined by analysis of the coupling constants comparisons with the known steroids.

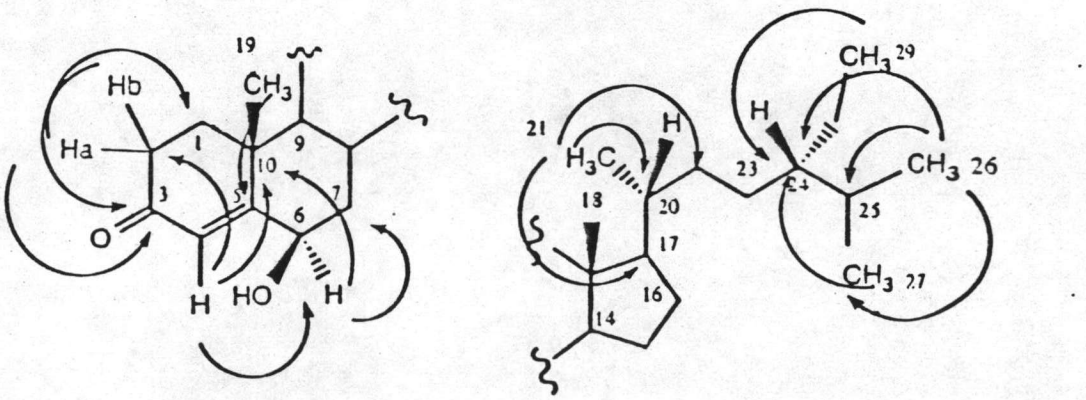


Figure 6. The long-range C-H correlation of compound M-060 observed from HMBC spectrum

Table 8. Carbon and proton chemical shift assignment, proton-proton correlations, and carbon proton long range correlations .

carbons	δ C (ppm)	δ H (ppm)	proton correlations in PDQF spectrum	long range correlations between ^{1}H and ^{13}C in HMBC spectrum
1	37.13	Ha, 2.04	Hb-1, Ha-2, Hb-2	C-2, C-10, C-19
		Hb, 1.71	Ha-1, Ha-2, Hb-2	C-2, C-9, C-10
2	34.28	Ha, 2.52	Hb-2, Ha-1, Hb-1	C-1, C-3
		Hb, 2.38	Ha-2, Ha-1, Hb-1	C-3
3	200.44	-	-	-
4	126.36	5.82	-	C-2, C-6, C-10
5	168.49	-	-	-
6	73.21	4.34	Ha-7, Hb-7	C-7, C-10
7	38.54	Ha, 2.00	Hb-7, H-6	-
		Hb, 1.23	Ha-7, H-6	-
8	29.76	1.95	H-9, H-14	-
9	53.66	0.91	H-8, 2H-11	-
10	38.02	-	-	-
11	21.01	1.49-1.54 (2 H)	H-9, Hb-12	C -8, C-9, C-12, C-13
12	39.63	Ha, 2.05	Hb-12	C-11, C-13, C-18
		Hb, 1.17	Ha-12, 2H-11	-
13	42.54	-	-	-
14	55.92	1.01	H-8, Ha-15	-
15	24.18	Ha, 1.62	Hb-15, H-14 Ha-16, Hb-16	-
		Hb, 1.15	Ha-15, Ha-16	-
16	28.18	Ha, 1.86	Hb-16, Ha-15	C-13
		Hb, 1.31	Ha-16,Ha-15	-
17	56.08	1.13	Ha-16	C-13, C-20
18	12.03	0.74	-	C-12, C-13, C-17
19	19.53	1.38	-	C-1, C-5, C-9, C-10

Table 8. (continue)

carbons	δ C (ppm)	δ H (ppm)	proton correlations PDQF spectrum	long range correlations between ^{1}H and ^{13}C in HMBC spectrum
20	36.26	1.38	-	-
21	18.80	0.93	-	C-17, C-20, C-22
22	33.91	Ha, 1.36	-	-
		Hb, 0.98	-	-
23	26.44	Ha, 1.32	Hb-23	-
		Hb, 1.04	Ha-23	-
24	46.10	0.94	-	-
25	29.00	1.68	H-26, H-27	C-26
26	19.00	0.81	H-25	C-24, C-25, C-27
27	19.61	0.83	H-25	C-24, C-25, C-26
28	23.06	Ha, 1.33	-	-
		Hb, 1.14	-	C-29
29	12.32	0.86	-	C-24, C-28

The relative configuration at C-6 can be assigned as 6α-H and 6β-OH by analysis of the H-6 splitting pattern. H-6 (δ 4.34 ppm) appears as a broad triplet and collapse into a triplet with the coupling constants 2.9 Hz after shaking with D_2O (Figure 16). The small magnitude of the coupling constant indicates the equatorial - axial and equatorial-equatorial relations between H-6 and the methylene protons at C-7.

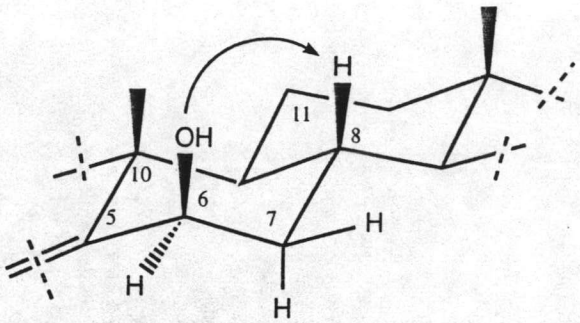
Procesterol [104], (Khan and Malik 1989), the known steroid with different configuration at C-6 (6β-H and 6α-OH) shows different splitting pattern to 2H-7s with the coupling constants 12.1 and 4.7 Hz. The carbon assignment of compond M-060 is similar to that of procesterol as summarized in Table 9.

Table 9. Carbon chemical shifts of Procesterol and M-060 (in $CDCl_3$)

Carbons	Procesterol	M-060
C-1	38.66	37.13
C-2	34.31	34.28
C-3	200.30	200.44
C-4	126.59	126.36
C-5	168.43	168.49
C-6	73.26	73.21
C-7	37.17	38.54
*C-8	45.93	29.76
C-9	53.72	53.66
C-10	38.08	38.02
C-11	21.04	21.01
C-12	39.68	39.63
C-13	42.59	42.54
C-14	56.79	55.92
C-15	24.32	24.18
C-16	28.22	28.18
C-17	56.16	56.08
C-18	11.92	12.03
C-19	19.84	19.53
C-20	36.28	36.26
C-21	18.78	18.80
C-22	33.99	33.91
C-23	26.43	26.44
C-24	46.11	46.10
C-25	29.01	29.00
C-26	19.09	19.00
C-27	19.59	19.61
C-28	23.16	23.06
C-29	12.32	12.32

However, C-8 of procesterol resonance at δ 45.93 ppm whereas C-8 of M-060 at δ 29.76 ppm. The upfield shift of C-8 in compound M-060 due to 1, 3 diaxial

effect of 6β-OH to the Hβ-8 [109]. This evidence confirm the proposed configuration at C-6.



[109]

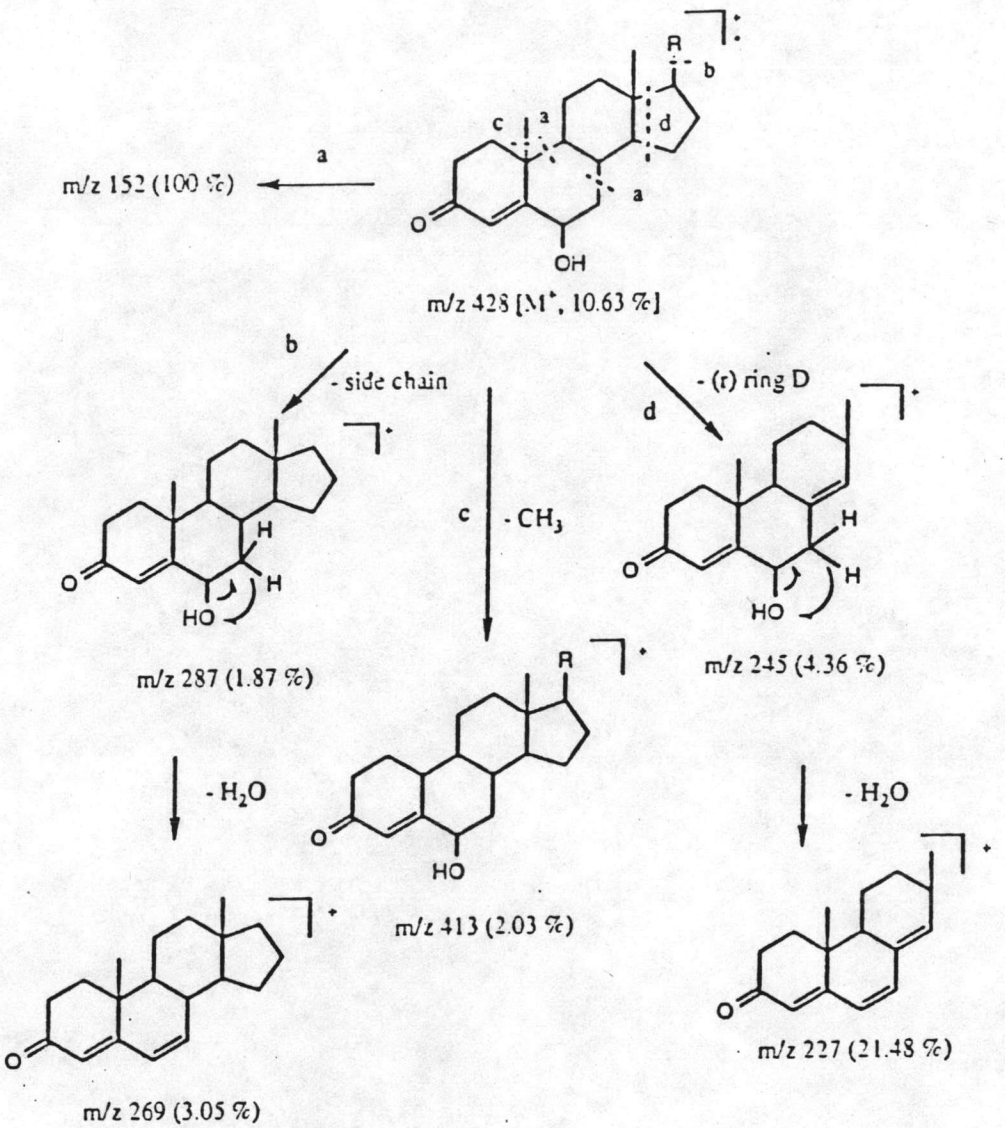
The absolute configuration at C-24 of compound M-060 is determined by ^{13}C nuclear magnetic resonance spectroscopy (Wright *et al.*,1978). The chemical shifts of side chain carbons of compound M-060 are compared with known C-24 ethyl epimers of steroids, 24 (*S*)-24-ethyl cholest-5-en-3β-ol and 24 (*R*)-24-ethyl cholest-5-en-3β-ol, data are shown in Table 10.

Table 10. Carbon chemical shifts of M-060, Compound 2*S*, and Compound 2*R* (in $CDCl_3$)

Carbons	M-060	Compound 2*S*	Compound 2*R*
20	36.26	36.29	36.17
21	18.80	18.82	18.82
22	33.91	33.95	33.95
*23	26.44	26.43	26.13
*24	46.10	46.07	45.85
*25	29.00	28.98	29.18
*26	19.00	19.07	19.84
*27	19.61	19.62	19.07
28	23.06	23.09	23.09
29	12.32	12.32	12.32

From data in Table 10, carbon-24 of M-060 can be assigned as 24*S* configurations. M-060 can be completely assign as a new steroidal ketone, (24 *S*)-24-ethylcholest-4-en-3-one-6β-ol or poriferast-4-en-3-one-6β-ol.

This structure is confirmed by analysis of the mass fragmentation (Scheme 3). The molecular ion fragment shows at m/z 428 (10.63 %). The cleavage of side chain shows fragment at m/z 287 (1.87 %). The cleavage of ring D with rearrangement shows fragment at m/z 245 (4.36 %). Subsequent loss of H_2O from this fragment shows fragment at m/z 227 (21.48 %).

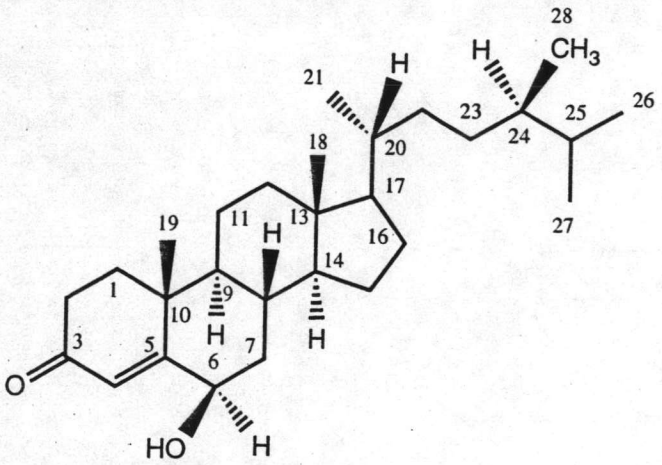


Scheme 3. Proposed mass fragmentation of M-060 (Budzikiewiez, 1972)

1.2 Compound M-059

Compound M-059, a white compound is obtained from fraction M-055 by semipreparative C-18 hplc column using 10 % water in methanol as a mobile phase. The uv absorption and ir spectrum of this compound are similar to compound M-060. This compound also shows uv absorption in chloroform (Figure 40) at λ_{max}. 243 (ε = 8238) which is characteristic of α, β unsaturated ketone. The ir spectrum (Figure 41) show absorption at 3,506 cm^{-1} (OH stretching), 1,676 cm^{-1} (C=O stretching), and 1,036 cm-1 (C-O stretching). The electron impact mass spectrum of compound M-059 (Figure 57) shows the molecular ion peak at m/z 414 (16.31 %) and establishs the tentative molecular formula of $C_{28}H_{46}O_2$. The molecular weight of this compound is 14 amu less than the molecular weight of compound M-060.

M-059 can be assigned as a new steroidal ketone, (24 *R*)-methylcholest-4-en-3-one-6β-ol or campest-4-en-3-one-6β-ol.[110] by analysis of its ^1H and ^{13}C nmr and comparing with M-060 [108], 22, 23-dihydro-brassicasterol [24 (S)-methyl], and 24 (*R*) -methyl cholesterol (Wright *et al.*, 1978).



[110]

The ^1H nmr spectrum of M-059 (Figure 42-45) shows six methyl groups. Splitting patterns of δ 1.37 ppm (19-CH_3) and δ 0.74 ppm (18-CH_3) are singlets which are similar to M-060. Other four methyl groups appear as doublets whereas one of four methyl groups of M-060 appear as a triplet. The proton signal at δ 0.86 ppm (29-CH_3 of M-060) shows triplet splitting pattern because of these protons coupling with two protons on C-28. Thus, M-059 is different from M-060 at the side chain (C-28 and C-29). The ^{13}C spectrum of M-059 shows only 28 carbons. Methyl group (C-28) of M-059 coupling with one proton on C-24 shows doublet splitting pattern. Four methyl groups at δ 0.92 ppm (d, J = 6.7 Hz), δ 0.85 ppm (d, J = 6.8 Hz), δ 0.81 ppm (d, J = 6.7 Hz), δ 0.78 (d, J = 6.7 Hz) are characteristic of 21-CH_3, 26-CH_3,

27-CH_3, and 28-CH_3 of C-28 steroid. In addition ^1H nmr spectrum also shows olefinic proton at δ 5.82 ppm (s) and proton attach to oxygenated carbon at δ 4.35 ppm (br.t, 2.8 Hz). These two protons are assigned as H-4 and H-6, respectively. Configuration of 6-hydroxyl group can be assigned as a β hydroxyl group by comparing with M-060.

The ^{13}C nmr spectrum of M-059 (Figures 46 and 47) shows 28 carbons which can be assigned as 4 quaternary carbons at δ 200.33 ppm, 168.37 ppm, 42.55 ppm, and 38.02 ppm, 9 methine carbons at δ 126.39 ppm, 73.36 ppm, 56.16 ppm, 55.93 ppm, 53.66 ppm, 38.87 ppm, 35.87 ppm, 32.45 ppm, 29.76 ppm, 10 methylene carbons at d 39.65 ppm, 38.61 ppm, 37.16 ppm, 34.29 ppm, 33.68 ppm, 30.33 ppm, 28.19 ppm, 24.18 ppm, 21.01 ppm, and 6 methyl carbons at δ 20.21 ppm, 19.53 ppm, 18.68 ppm, 18.28 ppm, 15.42 ppm, and 12.01 ppm. Cabons 1-19 of M-059 are assigned by comparing with M-060. The data are shown in Table 11.

Table 11. Carbon chemical shifts of M-059 and M-060 (in $CDCl_3$)

Carbons	M-059	M-060
C-1	37.16	37.13
C-2	34.29	34.28
C-3	200.33	200.44
C-4	126.39	126.36
C-5	168.37	168.49
C-6	73.36	73.21
C-7	38.61	38.54
C-8	29.76	29.76
C-9	53.66	53.66
C-10	38.02	38.02
C-11	21.01	21.01
C-12	39.65	39.63
C-13	42.55	42.54
C-14	55.93	55.92
C-15	24.18	24.18
C-16	28.19	28.18
C-17	56.16	56.08
C-18	12.04	12.03
C-19	19.54	19.53

Carbons 1-19 of M-059 can be comparing with carbons 1-19 of M-060 becauses of both are 4-en-3-one-6β-ol steroids. But, carbons C-20-C-28 of M-059 can not comparing with carbons C-20 to C-28 of M-060. Because of M-059 has methyl group substituted at C-24, whereas M-060 has an ethyl group substituted at C-24. Thus, chemical shift of side chain carbons of both compounds are differences. Side chain carbons of M-059 can be compared with 22, 23 dihydrobrassicasterol (compound 1S) and 24 (R) methyl cholesterol (compound 1R) (Wright *et al.*, 1978). The data are shown in Table 12.

Table 12. Carbon chemical shifts of M-059, compound 1S, compound 1R

Carbons	M-059	Compound 1S	Compound 1R
20	35.87	36.26	35.96
21	18.68	18.95	18.77
22	33.68	33.80	33.80
*23	30.33	30.67	30.37
*24	38.87	39.17	38.92
*25	32.45	31.54	32.49
*26	20.21	17.68	20.26
*27	18.28	20.56	18.32
28	15.42	15.51	15.44

According to data on Table 12, carbon 24 of M-059 can be assigned as 24 (R) configurations. Protons of M-059 can be assigned by using two dimensional nmr experiment, High Sensitive Quantum Coherence (HSQC). According to HSQC experiment (Figure 25-27), the directly coupled ^{1}H and ^{13}C are summarized in Table 13

Table 13. The carbon-proton correlations of M-059 observed in the HSQC spectrum

Carbons	δ (ppm)	Correlation with protons at δ (ppm)
C-1	37.16	2.04, 1.70
C-2	34.29	2.52, 2.38
C-4	126.39	5.82
C-6	73.36	4.35
C-7	38.61	2.00, 1.24
C-8	29.76	1.95

Table 13. (continue)

Carbons	δ (ppm)	Correlation with protons at δ (ppm)
C-9	53.66	0.91
C-11	21.01	1.47-1.54
C-12	39.65	2.05, 1.16
C-14	55.93	1.02
C-15	24.18	1.62, 1.14
C-16	28.19	1.86, 1.30
C-17	56.16	1.13
C-18	12.04	0.74
C-19	19.54	1.37
C-20	35.87	1.36
C-21	18.68	0.92
C-22	33.68	1.31, 1.06
C-23	30.33	1.14
C-24	38.87	0.78
C-25	32.45	1.53
C-26	20.21	0.85
C-27	18.28	0.81
C-28	15.42	0.78

The methylene carbon C-23 can be observed coupling with only one proton.

The Heteronuclear Multiple Bond Coherence (HMBC) spectrum (Figures 51-56) shows long range correlation between ^{1}H and ^{13}C. The long range ^{1}H and ^{13}C are shown on table 14. The correlation on rings A, B, C, and D of M-059 are similar to compound M-060. The connectivity of side chain is supported by the following correlation : H-17 (δ 1.13 ppm) and carbons C-20 (δ 35.87 ppm) and C-22 (33.68 ppm) ; 21-CH_3 (δ 0.92 ppm) and carbons C-17 (δ 56.16 ppm), C-20 (δ 35.87 ppm), C-22 (δ 33.68 ppm) ; 26-CH_3 (δ 0.85 ppm) and C-24 (δ 38.87 ppm), C-25 (δ 32.45 ppm), C-27 (δ 18.28 ppm) ; 27-CH_3 (δ 0.81 ppm) and C-24 (δ 38.87 ppm), C-25 (δ 32.45 ppm), C-26 (δ 20.21 ppm) ; 28-CH_3(δ 0.78 ppm) and C-23 (δ 30.33 ppm), C-24 (δ 38.87 ppm), C-25 (δ 32.45 ppm). The correlation on side chain show on Figure 7.

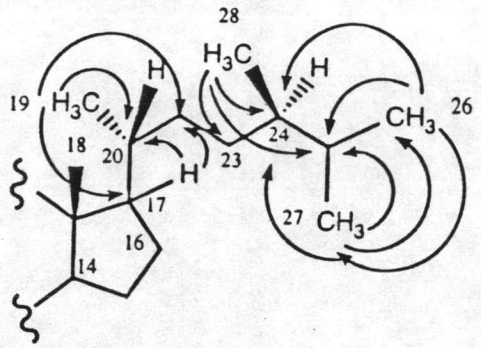


Figure 7. Side chain C-H long range correlation of M-059 observed in HMBC spectrum

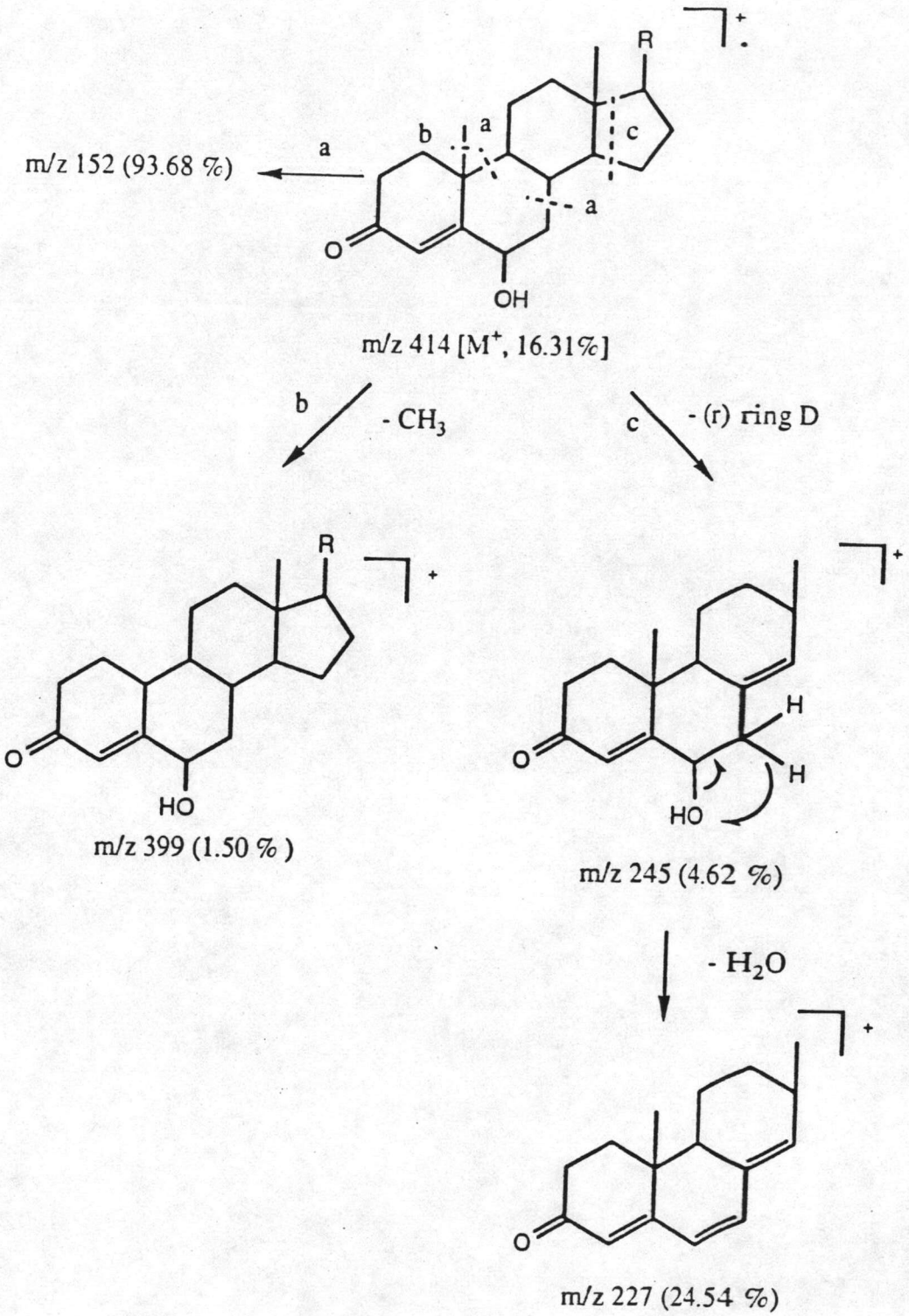
Table 14. Carbon and proton chemical shift assignment and carbon-proton long range correlations.

carbons	δ C (ppm)	δ H (ppm)	long range correlations between ^{1}H and ^{13}C in HMBC spectrum
1	37.16	2.04	C-2, C-9, C-10, C-19
		1.70	C-2, C-9, C-10, C-19
2	34.29	2.52	C-1, C-3
		2.38	C-1
3	200.33	-	-
4	126.39	5.82	C-2, C-6, C-10
5	168.37	-	-
6	73.36	4.35	-
7	38.61	2.00	-
		1.24	-
8	29.76	1.95	-
9	53.66	0.91	C-8
10	38.02	-	-
11	21.01	1.47-1.54	C-8, C-9, C-12
			C-13, C-19
12	39.65	2.05	C-11, C-13
		1.16	C-8, C-11, C-13
13	42.55	-	-
14	55.93	1.02	C-8, C-13, C-18
15	24.18	1.62	-
		1.14	C-16
16	28.19	1.86	C-13
17	56.16	1.13	C-8, C-12, C-13
			C-20, C-23
18	15.42	0.74	C-12, C-13, C-14, C-17
19	19.54	1.37	C-1, C-5, C-9, C-10
20	35.87	1.36	C-17, C-21, C-22
21	18.68	0.92	C-17, C-20, C-22

Table 14. (continue)

carbons	δ C (ppm)	δ H (ppm)	long range correlations between ^{1}H and ^{13}C in HMBC spectrum
22	33.68	1.31	-
		1.06	-
23	30.33	1.14	-
		-	-
24	38.87	0.78	-
25	32.45	1.53	-
26	20.21	0.85	C-24, C-25, C-27
27	18.28	0.81	C-24, C-25, C-26
28	12.04	0.78	C-23, C-24, C-25

This structure is confirmed by analysis of the mass fragmentation (Scheme 3). The molecular ion shows at m/z 414 (16.31 %). The cleavage of ring D with rearrangement shows fragment at m/z 245 (4.62 %). Subsequent loss of H_2O from this fragment shows at m/z 227 (24.54 %).



Scheme 4. Proposed mass fragmentation of M-059 (Budzikiewiez, 1972)

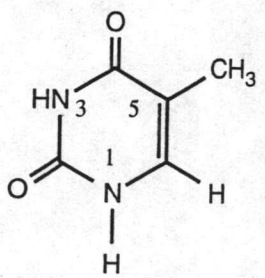
2. Structure elucidation of the isolated nucleosides

2.1 Compound A-044

Compound A-044, a white crystal, was obtained from fraction A-033 by flash column chromatographic technique using silica gel column (5 % methanol in chloroform) to yield 8 mg (4.9 x 10^{-3} % of crude aqueous extract). The uv spectrum of this compound (Figure 58) shows absorption at λ_{max} 264.5 nm (ε 9529), which is characteristic of pyrimidine base (Silverstein, Bassler, and Morrill 1991). The ir spectrum of A-044 (Figure 59) shows absorption 1,739 cm^{-1} and 1,676 cm^{-1} (carbonyl stretching). The electron impact mass spectrum (Figure 62) shows the molecular ion peak at m/z 126 (78.5%) estabishing the tentative molecular formula of $C_5H_6O_2N_2$.

A-044 can be assigned as a known pyrimidine base, thymine [111], by the analysis of its ^1H and ^{13}C-nmr spectra and comparing with A-047. The ^1H nmr spectrum of A-044 (Figure 60) shows the allylic coupling signals of one olefinic proton at δ 7.12 (q) ppm and one methyl group at δ 1.75 (d) ppm, with coupling constant 1.2 Hz. These protons are assigned as H-6 and CH_3 substituted on C-5, respectively.

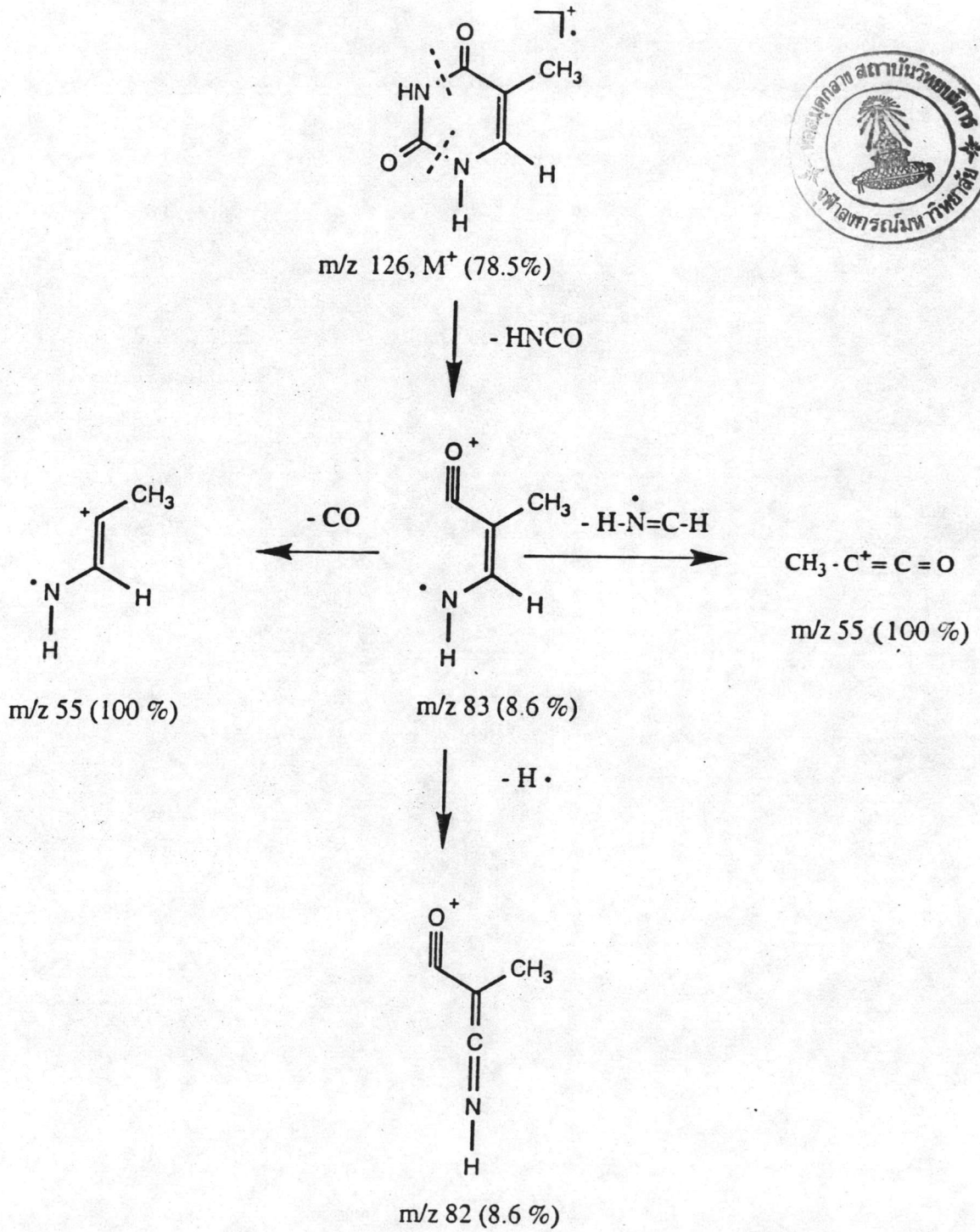
The ^{13}C nmr spectrum (Figure 61) shows two amide carbonyls at δ 167.46 ppm (C-4) and 153.73 ppm (C-2), sp^2 carbons at δ 139.14 (C-6) ppm and 110.46 ppm (C-5), and methyl carbon at δ 12.08 ppm (CH_3 substituted on C-5). The sructure of thymine [111] is shown as below.



[111]

This structure is confirmed by analysis of the mass fragmentation (Scheme 5). The molecular ion shows at m/z 126 (78.5%). The initial loss of HNCO

produces fragment ion at m/z 83 (8.6 %). This fragment ion subsequent loss of CO produces fragment ion at m/z 55 (100 %).



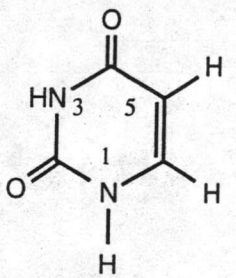
Scheme 5. Proposed mass fragmentations of compound A-044 (Hignite, 1972)

2.2 Compound A-046

Compound A-046, a white crystal was obtained from fraction A-033 by chromatographic technique using silica gel column (5 % methanol in chloroform) to yield 10 mg (6.1 x 10^{-3} % of crude aqueous extract). This compound is more polar than A-044. The uv spectrum of this compound (Figure 63) shows uv absorption at λ_{max} 259.3 nm (ε 6916), which is characteristic of pyrimidine base (Silverstein, Bassler, and Morrill 1991). The ir spectrum (Figure 64) exhibits absorption at 3,093 and 2,928 cm^{-1} (C-H stretching) and 1,734 and 1,645 cm^{-1} (carbonyl stretching). The electron impact mass spectrum (figure 67) shows the molecular ion peak at m/z 112 (100 %). Molecular formula should be $C_4H_4O_2N_2$.

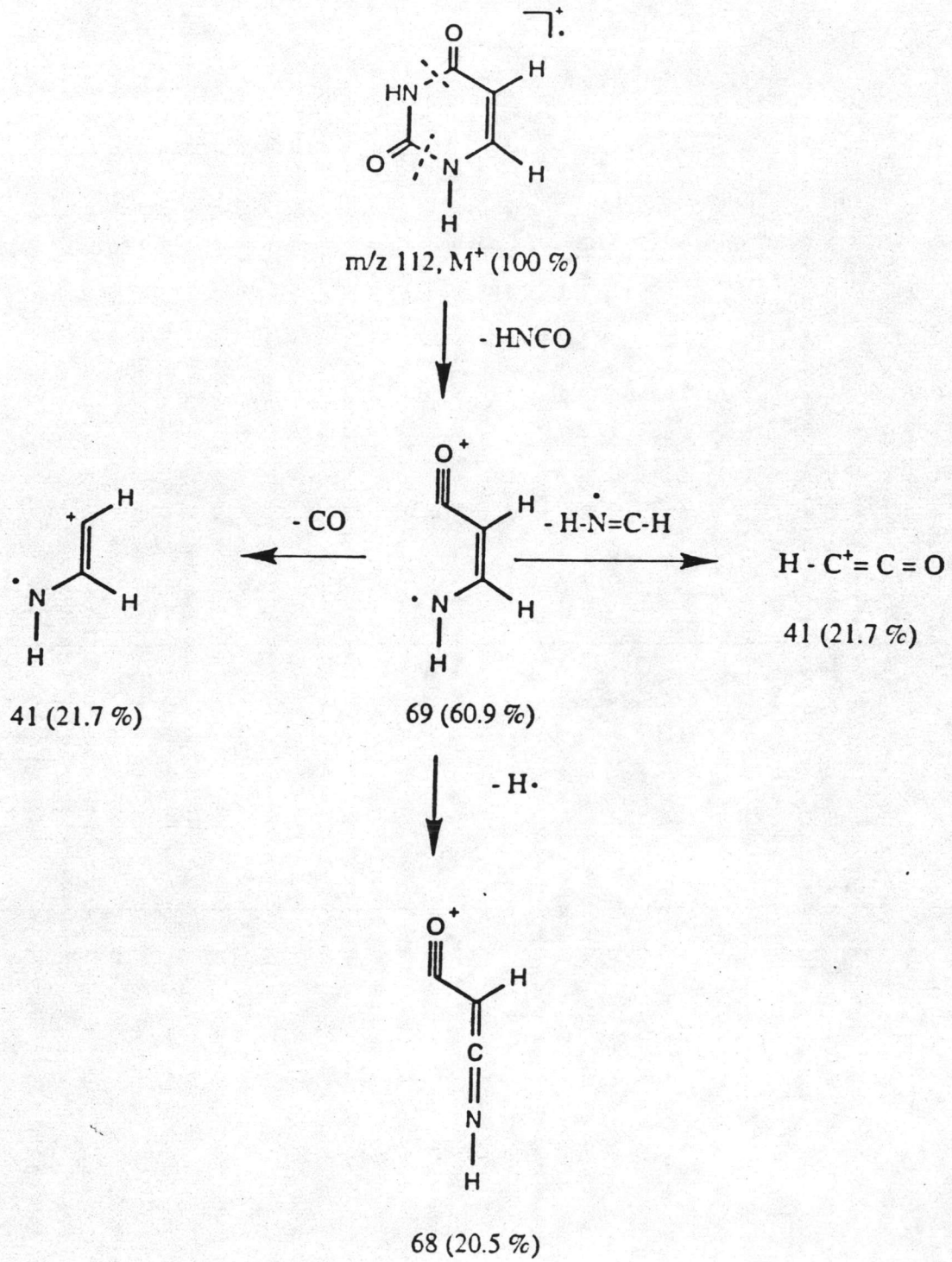
A-046 can be assigned as a known pyrimidine base, uracil [112]; by the analysis of ^1H and ^{13}C nmr spectra and comparing with A-049. The ^1H nmr spectrum of A-046 (Figure 65) shows signal of two olefinic protons at δ 7.24 and 5.52 ppm, coupled each other with coupling constant 7.6 Hz. These two protons are assigned as H-6 and H-5, respectively.

The ^{13}C nmr spectrum (Figure 66) shows two amide carbonyls at δ 167.31 ppm (C-4) and 153.52 ppm (C-2) and two sp^2 carbon at δ 143.49 ppm (C-6) and 101.73 ppm (C-5). The structure of uracil [112] is shown as below.



[112]

This structure is confirmed by analysis of the mass fragmentation (Scheme 6). The molecular ion shows at m/z 112 (100 %). The initial loss of HNCO produces fragment ion at m/z 69 (60.9 %). This fragment ion subsequent loss of CO produces fragment ion at m/z 41 (21.7 %).

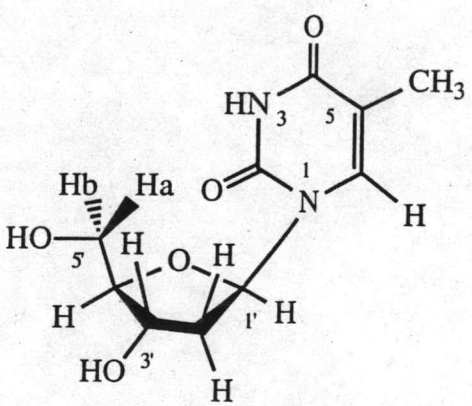


Scheme 6. Proposed mass fragmentations of compound A-046 (Hignite, 1972)

2.3 Compound A-047

Compound A-047 a white crystal, was obtained from fraction A-037 by chromatographic technique using Sephadex LH-20 (20% methanol in chloroform) to yield 17 mg (1.0 x 10^{-2} % of crude aqueous extract). This compound is more polar than A-044 and A-046. A-047 produces blue color with anisaldehyde sulfuric acid spraying reagent, since A-047 has a sugar in molecule. The uv spectrum of this compound (Figure 68) shows absorption at λ_{max} 266.4 nm (ε 10841), which characteristic of pyrimidine base (Silverstein, Bassler, and Morrill 1991). The ir spectrum (Figure 69) exhibits absorption at 3,322 cm^{-1} (OH stretching), 3,013 cm^{-1} (C-H stretching) and at 1,702 and 1,659 cm^{-1} (carbonyl stretching of amides). The electron impact mass spectrum (Figure 78) shows the molecular ion peak at m/z 242 (2.1%). Molecular formula should be $C_{10}H_{14}O_5N_2$.

A-047 can be assigned as a known pyrimidine nucleoside, thymidine; by the analysis of ^1H and ^{13}C nmr spectra. Molecular structure of thymidine [113] is shown as below.



[113]

The ^1H nmr spectrum of A-047 (Figure 70) shows one of olefinic proton, two methylene protons, three methine protons and one methyl group. The ^{13}C nmr spectrum (Figure 69) shows two carbonyl carbons two sp^2 carbons, four oxygenated sp^3carbons, and two sp^3 carbon.

The nucleoside base is observed by allylic coupling of the H-6 at δ 7.81 ppm (q) with the C-5 methyl protons at δ 1.88 ppm (d), with coupling constant 1.2 Hz.

The sugar part is observed by coupling of H-1' at δ 6.27 ppm (dd) to H-2'(β) and H-2'(α) with the coupling constants 6.7 and 6.7 Hz, respectively. The H-2'(β) at δ 2.20 ppm (ddd) shows coupling with H-2'(α), H-1', and H-3' with coupling constants 13.7, 7.4, and 6.1 Hz, respectively. The H-2'(α) at δ 2.25 ppm (ddd) shows coupling with H-2'(β), H-1', and H-3' with coupling constants 13.2, 6.3, and 3.8 Hz, respectively. The H-3' at δ 4.39 ppm (dt) shows coupling with H-2'(β), H-2'(α), and H-4', with coupling constants 6.1, 3.3, and 3.3 Hz, respectively. The H-4' at δ 3.90 ppm.(q) shows coupling with H-3' and 2H-5', with coupling constants 3.4 and 3.4 Hz, respectively. The Ha-5' at δ 3.73 ppm.(dd) shows coupling with Hb-5' and H-4', with coupling constants 12.0 and 3.6 Hz. The Hb-5' at δ 3.80 ppm.(dd) shows coupling with Ha -5' and H-4', with coupling constants 12.0 and 3.2 Hz, respectively.

The complete carbon assignments of compound A-047 are achieved by the analysis of the ^{1}H-detected High Sensitive Quantum Coherence (HSQC) and ^{1}H-detected Heteronuclear Multiple Bond Quantum Coherence (HMBC) spectra, which provide the correlations between protons and carbons through one bond and long range coupling, respectively.

According to HSQC spectrum (Figure 72), the protonated carbons are assigned as follows; 3H of methyl group (δ 1.88 ppm) - 5-CH_3 (δ 12.47 ppm), H-6 (δ 7.81ppm) - C-6 (δ 138.21 ppm), H-1' (δ 6.27 ppm) - C-1' (δ 86.30 ppm), H-2' (δ 2.25 ppm and δ 2.20 ppm) - C-2' (δ 41.24 ppm), H-3' (δ 4.39 ppm) - C-3' (δ 72.25 ppm), H-4' (δ 3.90 ppm) - C-4' (δ 88.87 ppm), H-5' (δ 3.73 ppm and 3.80 ppm) - C-5' (δ 62.89 ppm).

The quaternary carbons assignment are determined by the long range coupling observed from the HMBC spectrum (Figure 73). Two signals from carbon spectrum at the down field region are the amide carbons. The signal at δ 166.45 ppm is assigned as C-4 by the long range coupling with H-6 (δ 7.81 ppm) and 5-CH_3 (δ 1.88 ppm). The signal at δ 152.42 ppm is assigned as C-2 by the long range coupling with H-6 (δ 7.81 ppm) and H-1' (δ 6.27 ppm). There is long range correlation between C-2 of pyrimidine base and H-1' of sugar part, but no long range correlation between C-4 of pyrimidine base and H-1' of sugar part, confirm that the sugar part substitute on N-1. There is one remained quaternary carbon C-5 at δ 111.57 ppm confirm by long range correlation with H-6 (at δ 7.81 ppm) and 5-CH_3 (at δ 1.88).

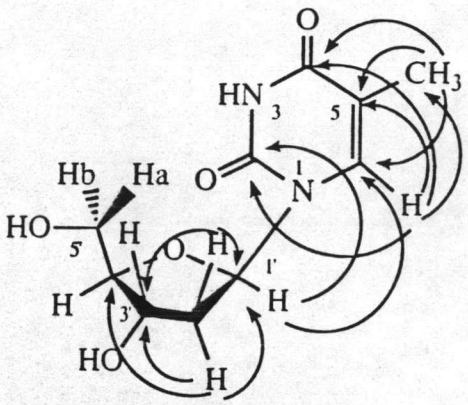


Figure 8. The long range C-H correlation of compound A-047 observed in HMBC spectrum

The relative configuration of the sugar part is proposed by Nuclear Overhauser Effect (NOE) difference spectrum (Table). The NOE shows correlation among protons through space, by increasing intensity of proton signals which are correlated to the irradiated protons. Generally, the base of nucleoside is β oriented and H-1' is α oriented.

Table 15. NOE enhancements for compound A-047

Irradiated protons	% NOE						
	H-1'	Hα-2'	Hβ-2'	H-3'	H-4'	Ha-5'	H-6
H-1'	-	7.6	-	-	2.6	-	2.8
Hβ-2'	9.2	-	-	14.3	-	-	13.9
H-3'	-	-	9.2	-	3.4	2.0	1.7
H-6	5.1	-	4.3	2.6	-	-	-

H-3' (δ = 4.39 ppm) and one of the two H-2's (δ = 2.20 ppm) proved to be the same side as the nucleoside base (β-oriented) by the following results: the 2.6 % NOE at H-3' and the 4.3 % NOE at H-2' upon irradiation of H-6 (δ = 7.81 ppm, Figure 74), the 14.3 % NOE at H-3' upon irradiation of H-2' (δ 2.20 ppm, Figure 77) and the 9.2 % NOE at Hβ-2' and 2.0 % NOE at Ha-5' (δ 3.73 ppm) upon irradiation of H-3' (δ 4.39 ppm, Figure 76)

H-4' (δ 3.90 ppm), one of the two H-2's (δ = 2.25 ppm) and H-1' (δ 6.27 ppm) are situated on the same face of the ring (α oriented) based on the following evidence : the 2.6 % NOE at H-4' and the 7.6 % NOE at H-2' upon irradiation of H-1' (Figure 75).

The close proximities of H-3', H-4' and of H-1', Hβ-2' through the quasiaxial and quasiequatorial orientations of the tetrahydrofuran ring are observed by the % enhancement of these protons such as 3.4 % NOE at H-4' upon irradiation of H-3' and Hβ-2' upon irradiation of H-1'.

The 2.9 %, 13.9 %, and 1.7 % enhancement of H-6 signal intensity upon irradiation of H-1', H-2' and H-3' suggest that two orientations (I, II) of the pyrimidine base exist as shown. H-6 is facing outward the sugar part in from I while facing inward the sugar part in form II. Because of the rotation of C-1'-N-1 bond, these two form are quickly interconversion.

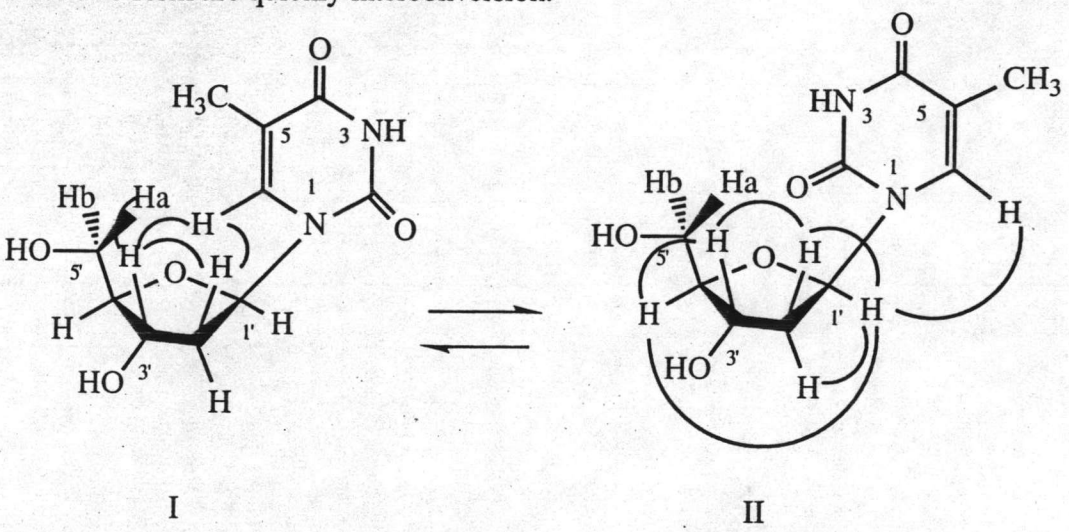
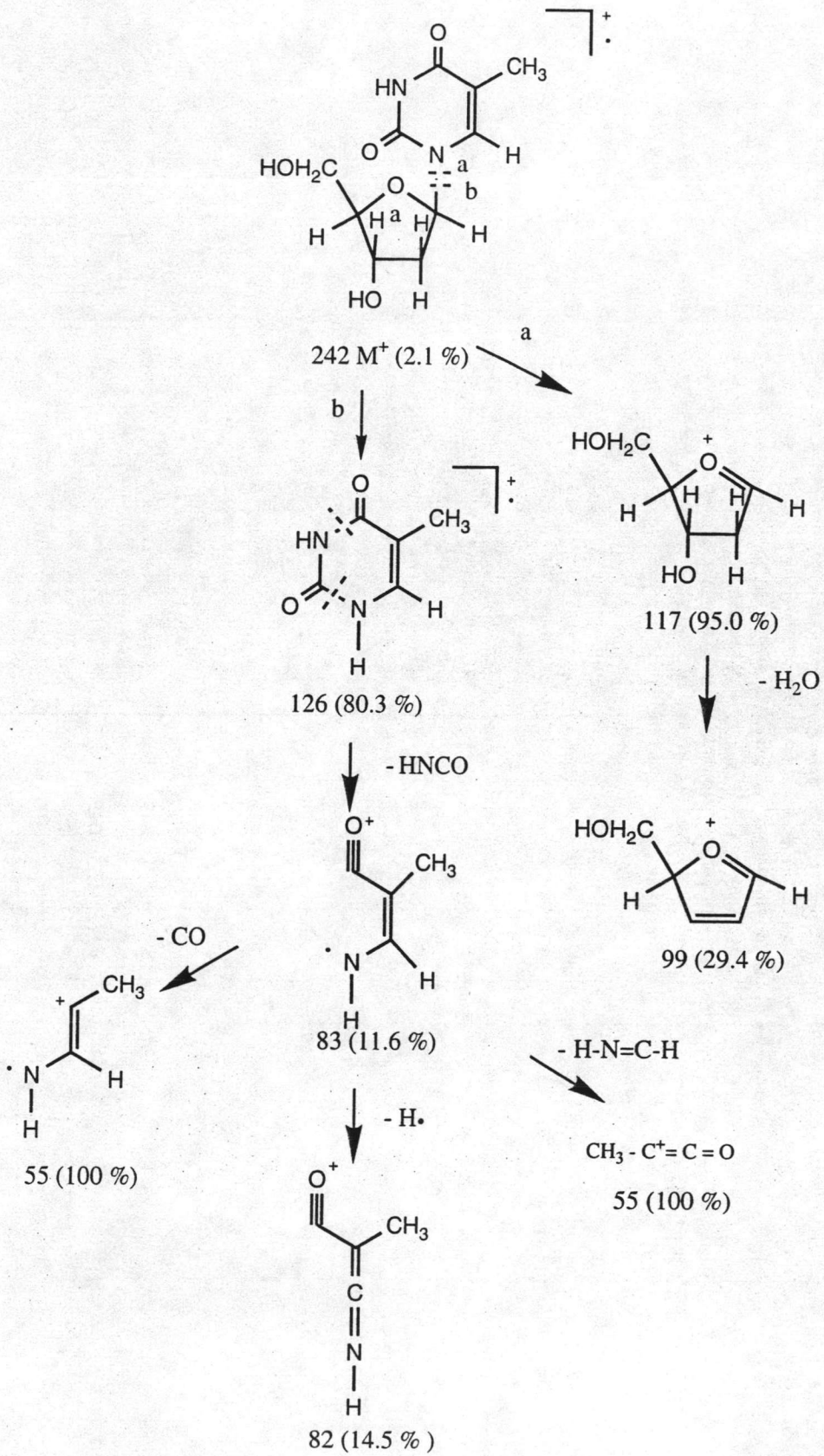


Figure 9. Result from NOE experiment of compound A-047

This structure is confirmed by analysis of the mass fragmentation (Scheme 7). The α cleavage o C-N bond provides the fragment of sugar moeity m/z 117 (95.0 %). The αα cleavage and rearrangement provides the fragment of pyrimidine base m/z 126 (80.3 %).

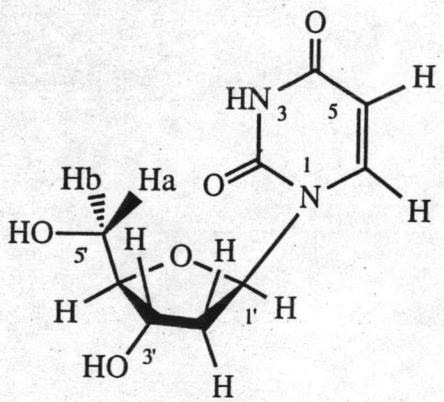


Scheme 7. Proposed mass fragmentations of compound A-047 (Hignite, 1972)

2.4 Compound A-049

Compound A-049, a white crystal was obtained from fraction A-037 by chromatographic technique using Sephadex LH-20 (20% methanol in chloroform) to yield 20 mg (1.2 x 10^{-2} % of crude aqueous extract). This compound is more polar than A-047. Compound A-049 produces blue color with anisaldehyde sulfuric acid spraying reagent, since A-049 also has a sugar in molecule. The uv spectrum of this compound (Figure 80) absorption at λ_{max} 262.3 nm (ε 9,450), which characteristic of pyrimidine base (Silverstein, Bassler, and Morrill 1991). The ir spectrum (Figure 81) exhibits absorption at 3,269 cm^{-1} (OH stretching), 1,701 and 1,674 cm^{-1} (carbonyl strteching). The electron impact mass spectrum (Figure 85) shows the molecular ion peak at m/z 228 (< 1%). Molecular formula should be $C_9H_{12}O_5N_2$.

A-049 can be assigned as a known pyrimidine nucleoside, 2' deoxy uridine; by the analysis of ^1H and ^{13}C nmr spectra and comparing with A-047. Molecular structure of 2' deoxy uridine [114] is shown as below.



[114]

The ^1H nmr spectrum of A-049 (Figure 82) shows two of olefinic protons, four methylene protons, and three methine protons. The ^{13}C nmr spectrum (Figure 83) shows two amide carbonyl carbons two sp^2 carbons, four oxygenated sp^3 carbons, and one sp^3 carbon.

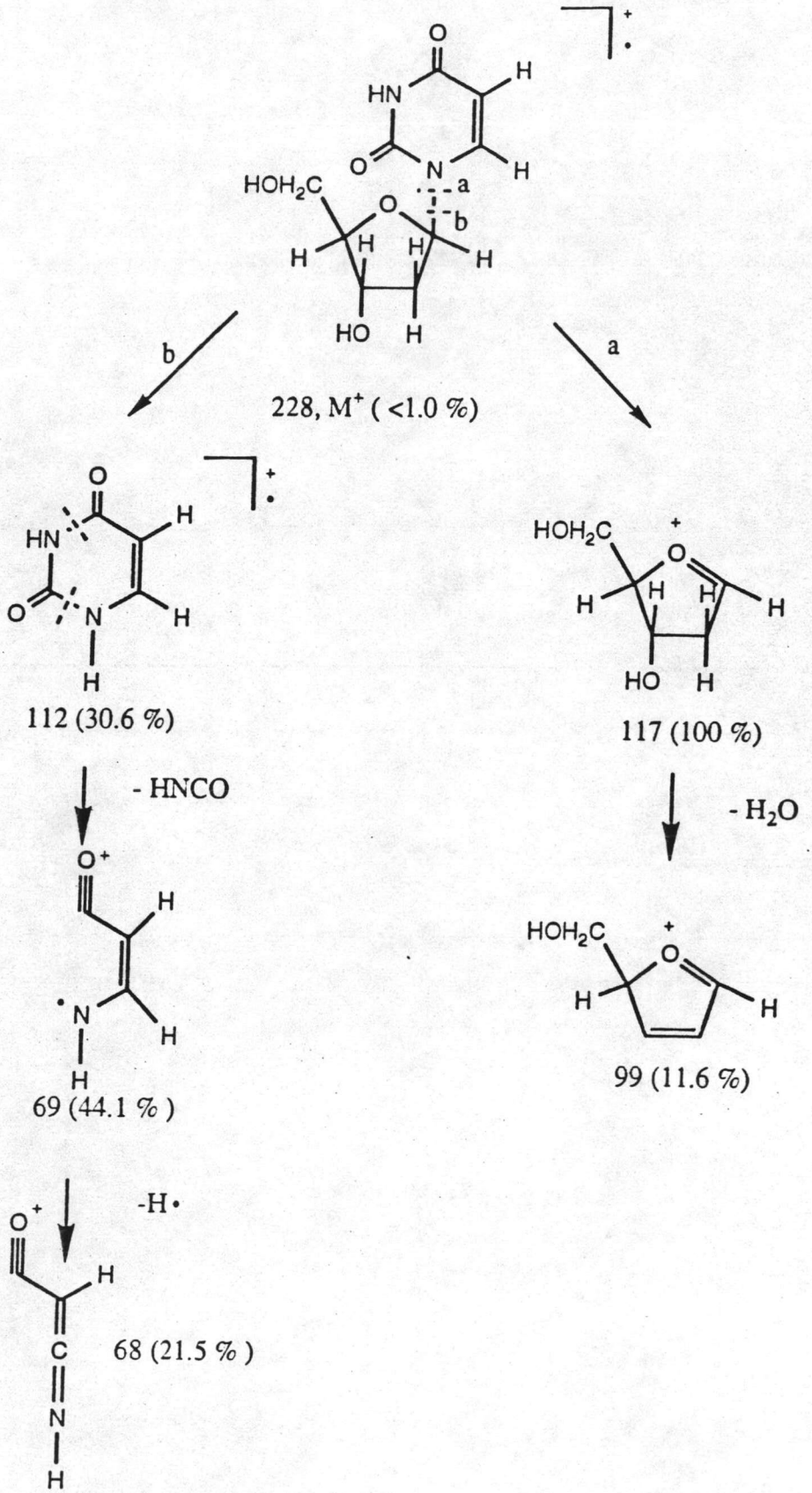
The nucleoside base is observed by coupling of the H-6 at δ 7.97 (d) ppm with H -5 at δ 5.69 (d) ppm), with coupling constant 8.2 Hz.

The sugar part is observed by coupling of H-1' at δ 6.26 (dd) ppm with H-2'(β) and H-2'(α) at δ 2.20 and 2.29 ppm with the coupling constants 6.7 and 6.7 Hz, respectively. The H-2'(β) at δ 2.20 ppm (ddd) shows coupling with H-2'(α), H-1', and H-3' with coupling constants 13.6, 7.3, and 6.1 Hz, respectively. The H-2'(α) at δ 2.29 ppm (ddd) shows coupling with H-2'(β), H-1', and H-3' with coupling constants 13.4, 6.3, and 3.5 Hz, respectively. The H-3' at δ 4.38 (dt) ppm shows coupling with H-2' (β), H-2' (α), and H-4', with coupling constants 6.1, 3.4, and 3.3 Hz, respectively. The H-4' at δ 3.92 (q) ppm shows coupling with H-3' and 2H-5', with coupling constants 3.4 and 3.4 Hz. The Ha-5' at δ 3.72 ppm.(dd) shows coupling with Hb-5' and H-4', with coupling constants 12.0 and 3.6 Hz. The Hb-5' at δ 3.78 (dd) ppm shows coupling with Ha -5' and H-4', with coupling constant 11.9 and 3.4 Hz, respectively.

The complete carbon assignments are achived by the analysis of the ^{1}H-detected High Sensitive Quantum Coherence (HSQC), which provide the correlations between protons and carbons through one bond and long range coupling, respectively.

According to HSQC spectrum (Figure 84), the protonated carbons are assigned as followed; H-5 (δ 5.69 ppm) - C-5 (δ 102.69 ppm), H-6 (δ 7.97ppm) - C-6 (δ 142.56 ppm), H-1' (δ 6.26 ppm) - C-1' (δ 86.68 ppm), H-2' (δ 2.29 ppm and δ 2.20 ppm) - C-2' (δ 41.41 ppm), H-3' (δ 4.38 ppm) - C-3' (δ 72.29 ppm), H-4' (δ 3.92 ppm) - C-4' (δ 89.03 ppm), H-5' (δ 3.72 ppm and 3.78 ppm) - C-5' (δ 62.89 ppm). Carbons at δ 152.26 ppm and 166.27 ppm are assigned as C-2 and C-4 by comparing with A-047.

This structure is confirmed by analysis of the mass fragmentation (Scheme 8). The α cleavage of C-N bond provides the fragment of sugar moeity m/z 117 (100.0 %). The αα cleavage and rearrangement provide the fragment of pyrimidine base m/z 112 (30.6 %). The pyrimidine base further loss of HNCO produce fragment at m/z 69 (44.1 %)



Scheme 8. Proposed mass fragmentations of compound A-049 (Hignite, 1972)

^{1}H and ^{13}C assignments of compounds A-044, A-046, A-047, and A-049 are summarized on the Table 16 and Table 17.

Table 16. ^{1}H assignments of compounds A-044, A-046, A-047, and A-049 (in CD_3OD)

Position	δ H (ppm) and J (Hz)			
	A-044	A-046	A-047	A-049
5	-	5.52 (d, 7.6)	-	5.69 (d, 8.2)
5-CH_3	1.75 (d, 1.2)	-	1.88 (d, 1.2)	-
6	7.12 (q, 1.2)	7.24 (d, 7.6)	7.81 (q, 1.2)	7.97 (d, 8.2)
1'	-	-	6.27 (dd, 6.7,6.7)	6.26 (dd, 6.7, 6.7)
2' (α)	-	-	2.25 (ddd, 13.2, 6.3, 3.8)	2.29 (ddd, 13.4, 6.3,3.5)
2' (β)	-	-	2.20 (ddd, 13.7, 7.4, 6.1)	2.20 (ddd, 13.6, 7.3, 6.1)
3'	-	-	4.39 (dt, 6.1, 3.3)	4.38 (dt, 6.1, 3.3)
4'	-	-	3.90 (q, 3.4)	3.92 (q, 3.4)
5'a	-	-	3.73 (dd, 12.0, 3.6)	3.72 (dd, 12.0, 3.6)
5'b	-	-	3.80 (dd, 12.0, 3.2)	3.78 (dd, 11.9, 3.4)

Table 17. ^{13}C assignments of compounds A-044, A-046, A-047, and A-049 (in CD_3OD)

Position	δ C (ppm)			
	A-044	A-046	A-047	A-049
2	153.73	153.52	152.42	152.26
4	167.46	167.31	166.45	166.27
5	110.46	101.73	111.57	102.69
CH_3-5	12.08	-	12.47	-
6	139.14	143.49	138.21	142.56
1'	-	-	86.30	86.68
2'	-	-	41.24	41.41
3'	-	-	72.25	72.29
4'	-	-	88.87	89.03
5'	-	-	62.89	62.89